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Population-Based Survey of Filamentous Fungi and Antifungal Resistance in Spain (FILPOP Study)

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A population-based survey was conducted to investigate the epidemiology of and antifungal resistance in Spanish clinical strains of filamentous fungi isolated from deep tissue samples, blood cultures, and respiratory samples. The study was conducted in two different periods (October 2010 and May 2011) to analyze seasonal variations. A total of 325 strains were isolated in 29 different hospitals. The average prevalence was 0.0016/1,000 inhabitants. Strains were identified by sequencing of DNA targets and susceptibility testing by the European Committee for Antimicrobial Susceptibility Testing reference procedure. The most frequently isolated genus was *Aspergillus*, accounting for 86.3% of the isolates, followed by *Scedosporium* at 4.7%; the order Mucorales at 2.5%; *Penicillium* at 2.2%, and *Fusarium* at 1.2%. The most frequent species was *Aspergillus fumigatus* (48.5%), followed by *A. flavus* (8.4%), *A. terreus* (8.1%), *A. tubingensis* (6.8%), and *A. niger* (6.5%). Cryptic/sibling *Aspergillus* species accounted for 12% of the cases. Resistance to amphotericin B was found in 10.8% of the isolates tested, while extended-spectrum triazole resistance ranged from 10 to 12.7%, depending on the azole tested. Antifungal resistance was more common among emerging species such as those of *Scedosporium* and Mucorales and also among cryptic species of *Aspergillus*, with 40% of these isolates showing resistance to all of the antifungal compounds tested. Cryptic *Aspergillus* species seem to be underestimated, and their correct classification could be clinically relevant. The performance of antifungal susceptibility testing of the strains implicated in deep infections and multicentric studies is recommended to evaluate the incidence of these cryptic species in other geographic areas.

The number of fungal pathogenic species has increased significantly in recent years (1, 2). The increase in the size of the population at risk of fungal infections and the advances in diagnostic tools have been pointed out as possible reasons for this increase. As the population at risk is expected to keep growing in the coming years, the interest in the epidemiology of fungal infections and the taxonomy of the relevant fungi is also increasing. The use of molecular tools for fungal identification has allowed a deeper study of pathogenic fungal genetics, and as a consequence, several species have been revealed to be species complexes (3–5). They are formed by species that are almost indistinguishable by morphological methods; hence, they have been designated cryptic species. Therefore, classical identification methods that rely on phenotypic characteristics are no longer suitable for strain classification and the use of molecular tools is continuously yielding descriptions of new taxa (3, 6, 7).

Some of these species have already been found in clinical samples, but their prevalence and relevance in the clinical setting are still unknown. Several studies on the epidemiology of yeast infections have been published (8–10), but the limited available data on molds are based mainly on retrospective studies or deal only with specific groups of molds (11–14). In addition, according to some clinical trials, 50 to 75% of the patients enrolled are diagnosed by the microscopic examination of tissues or by the detection of fungal components, which means that the species causing the infection is never known in a significant number of cases (15, 16). However, the prevalence of rare, emerging, cryptic, and sibling species seems to be rising. Species of genera such as *Scedosporium* and *Fusarium* could cause 5 to 10% of the deep mycoses in some geographic areas (17, 18). Recently, the frequency of cryptic species of molds has been analyzed in two studies of transplant patients in the United States (19, 20). To our knowledge, the prevalence of these species has not being studied in Europe so far. In addition, some of these cryptic species, such as *Aspergillus lentulus* and *A. calidoustus*, are more resistant to the antifungal drugs available (3, 6), highlighting the importance of correct identification. Moreover, some studies have pointed out the emergence of secondary resistance in *Aspergillus* species in Europe (21, 22), but its prevalence in Spain has not been investigated yet.

In this study, we analyzed the species distribution and prevalence of antifungal drug resistance in Spain through a multicenter prospective study involving 29 hospitals in different regions of Spain.

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MATERIALS AND METHODS

Strains. This study was conducted prospectively in two different seasons, fall (October 2010) and spring (May 2011). We included all of the patients admitted to 29 Spanish hospitals who were culture positive for filamentous fungi on the basis of respiratory samples, blood cultures, or biopsy specimens. The strains were sent to the Spanish National Center of Microbiology for identification and susceptibility testing. A referral form was filled out for each isolate and included demographic and clinical data. Strains were classified as colonizers or as being of clinical relevance

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(proven, probable, and suspected infections) according to the site of isolation and the clinical report (23).

The prevalence of fungal infections by filamentous fungi was calculated for each hospital by using as the reference the number of patients admitted during each period divided by the average population associated with the hospital (data provided by the hospitals).

Morphological identification. The strains were subcultured in different media to ascertain their macroscopic and microscopic morphology. The media included malt extract agar (2% malt extract [Oxoid S.A., Madrid, Spain]), potato dextrose agar (Oxoid S.A.), oatmeal agar (Oxoid S.A.), potassium chloride agar (Oxoid S.A.), and Czapek-Dox agar (Difco, Soria Melguizo S.A., Madrid, Spain). Cultures were incubated at 30 and 37°C. Fungal morphological features were examined macro- and microscopically by conventional methods (24).

Molecular identification. Molds were subcultured in GYEP medium (0.3% yeast extract, 1% peptone; Difco, Soria Melguizo) with 2% glucose (Sigma-Aldrich Química, Madrid, Spain) for 24 to 48 h at 30°C. Genomic DNA was isolated by a previously described extraction procedure (25). Molecular identification was performed by sequencing informative targets. DNA segments comprising the internal transcribed spacer 1 (ITS1) and ITS2 regions of all of the strains were amplified with primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (26). For *Aspergillus* and *Scedosporium* isolates, a portion of the β-tubulin gene was sequenced with primers βtub3 (5′-TTCACCTTCAGACCGGT-3′) and βtub2 (5′-AGTTGTCGGGACGGAATAG-3′) (3) for *Aspergillus* and primers TUB-F (5′-CTGTCCAACCCCTCTTACGGCGACCTGAAC-3′) and TUB-R (5′-ACCCTCACCAGTATACCAATGCAAGAAAGC-3′) (27) for *Scedosporium*. Also, DNA segments of *Fusarium* isolates comprising the elongation factor alpha region were amplified with primers EF1 (5′-ATGGGTAAGARGACAAGAC-3′) and EF2 (5′-GGARGTACCAGTSATCATGTT-3′) (28). All of the primers were synthesized by Sigma Genosys (Madrid, Spain). The reactions were performed in a GeneAmp PCR System 9700 (Applied Biosystems). The reaction mixtures contained 0.5 μM each primer, 0.2 μM each deoxynucleoside triphosphate, 5 μl of PCR 10× buffer (Applied Biosystems, Madrid, Spain), 2.5 U of *Taq* DNA polymerase (AmpliTaq; Applied Biosystems), and 25 ng of DNA in a final volume of 50 μl. The samples were amplified in a GeneAmp PCR System 9700 (Applied Biosystems) by using the following cycling parameters: 1 initial cycle of 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 45 s at 56°C (ITS), 55°C (β-tubulin) or 47°C (elongation factor α), and 2 min at 72°C, with 1 final cycle of 5 min at 72°C. The reaction products were analyzed in a 0.8% agarose gel. Sequencing reactions were done with 2 μl of a sequencing kit (BigDye Terminator cycle sequencing ready reaction; Applied Biosystems), 1 μM primers (the same as in the PCR, except that for *Aspergillus* β-tubulin, βtub1 [5′-AATTGGTGCCGCTTTCTGG-3′] and βtub4 [5′-AGCGTCCATGGTACCAGG-3′] were used), and 3 μl of the PCR product in a final volume of 10 μl.

Sequences were assembled and edited with the SeqMan II and EditSeq software packages (Lasergene; DNAStar, Inc., Madison, WI). All of the sequences were compared with reference sequences from the GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) and Mycobank (http://www.mycobank.org/) databases with InfoQuest FP software, version 4.50 (Bio-Rad Laboratories, Madrid, Spain), as well as with the database belonging to the Department of Mycology of the Spanish National Center for Microbiology, which holds 9,000 sequences from strains belonging to 270 different fungal species. This database was designed by the Spanish National Center for Microbiology, and access to it is restricted.

Antifungal susceptibility testing. Microdilution testing was performed in accordance with the European Committee for Antimicrobial Susceptibility Testing (EUCAST) standard method (29). *Aspergillus fumigatus* ATCC 2004305 and *Aspergillus flavus* ATCC 2004304 were used as quality control strains. The antifungal agents used in this study were amphotericin B (Sigma-Aldrich Química), itraconazole (Janssen Pharmaceutica, Madrid, Spain), voriconazole (Pfizer S.A., Madrid, Spain), ravuconazole (Bristol-Myers Squibb, Princeton, NJ), posaconazole (Schering-Plough Research Institute, Kenilworth, NJ), terbinafine (Novartis, Basel, Switzerland), caspofungin (Merck & Co., Inc., Rahway, NJ), micafungin (Astellas Pharma Inc., Tokyo, Japan), and anidulafungin (Pfizer S.A.). The final concentrations tested ranged from 0.03 to 16 mg/liter for amphotericin B, terbinafine, caspofungin, micafungin, and anidulafungin and from 0.015 to 8 mg/liter for itraconazole, voriconazole, ravuconazole, and posaconazole. Plates were incubated at 35°C for 48 h in a humidified atmosphere. Visual readings were performed at 24 and 48 h with the help of a mirror. The endpoint for amphotericin B, itraconazole, voriconazole, ravuconazole, posaconazole, and terbinafine was the antifungal concentration that produced complete inhibition of visual growth at 24 and 48 h. For the echinocandins, the endpoint was the antifungal concentration that produced a visible change in the morphology of the hyphae compared with the growth control well (minimum effective concentration). The EUCAST has set breakpoints for the interpretation of antifungal susceptibility testing results for amphotericin B (resistant strain MIC, >2 mg/liter), itraconazole (MIC, >2 mg/liter), voriconazole (MIC, >2 mg/liter), and posaconazole (MIC, >0.25 mg/liter) (http://www.eucast.org/clinical_breakpoints/) (29–31). These breakpoints have been set for only some *Aspergillus* species but were used in this study to analyze the rates of *in vitro* resistance of all of the species. Breakpoints of echinocandins have not been set yet, and rates of resistance were not calculated.

Statistical analysis. Descriptive and comparative analyses were done. Differences in the proportions of fungal species were determined by Fisher's exact test or by chi-square analysis. The significance of the differences between MICs was determined by analysis of variance (with Bonferroni's *post hoc* test) or by nonparametric tests. $P < 0.01$ was considered statistically significant. Statistical analysis was performed with IBM SPSS Statistics 19.0 (SPSS Iberica, Madrid, Spain).

RESULTS

The average prevalence was 0.002% (number of isolates divided by number of admitted patients) or 0.017/1,000 inhabitants in October 2010 and 0.0018% or 0.016/1,000 inhabitants in May 2011. A total of 325 isolates from 23 hospitals were included in this study. Two hundred seven isolates were collected in the first period (October 2010), and 118 were collected in the second (May 2011). Six hospitals reported no isolates matching the conditions of the study. A total of 309 (95%) of the 325 clinical strains were isolated from respiratory samples. Of these 309 isolates, 186 (60%) were regarded as colonizers. The remaining 123 isolates (40%) were counted as being of clinical relevance, including 32/123 (26%) recovered from bronchoalveolar lavage fluid samples. Fungi were cultured from tissue samples or sterile fluid samples from 5% of the patients (16/325). Proven infections were reported in 13 cases, including 1 of those with positive blood cultures. Three cases with isolates recovered by drainage of deep sites were regarded as colonization.

Of the 325 isolates included in this study, 322 were identified by the observation of morphologic characteristics, ITS sequencing, and part of the β-tubulin or elongation factor α gene when needed; three isolates could not be analyzed at the reference center because of absence of growth or contamination when received. Table 1 shows the number of isolates classified in each genus. The most frequently found genus was *Aspergillus*, accounting for 86.3% (278 isolates) of the isolates found, followed by *Scedosporium* at 4.7% (15 isolates), the order Mucorales at 3.7% (12 isolates), *Penicillium* at 2.2% (7 isolates), and *Fusarium* at 1.2% (4 isolates). Table 1 also displays the isolation rates of genera by clinical significance (colonizers versus of clinical relevance). No statistically significant differences were found, although researchers reported members of the order Mucorales as being of clinical rel-

TABLE 1 The genera most commonly isolated in the FILPOP study, the numbers and percentages of isolates, and their clinical relevance according to researchers' reports

Genus or order	No. (%) of strains clinically relevant	No. (%) of colonizers	Total no. % of isolates in FILPOP study
Aspergillus	117 (86.6)	161 (86.1)	278 (86.3)
Scedosporium	5 (3.7)	10 (5.3)	15 (4.7)
Mucorales	8 (6.0)	4 (2.2)	12 (3.7)
Penicillium	2 (1.5)	5 (2.6)	7 (2.2)
Fusarium	1 (0.7)	3 (1.6)	4 (1.2)
Other[a]	2 (1.5)	4 (2.2)	6 (1.9)
Total	135 (100.0)	187 (100.0)	322[b] (100.0)

[a] The other species (one each) belonged to the genera *Arthrinium*, *Psathyrella*, *Cladosporium*, *Purpureocillium*, *Phialemonium*, and *Scopulariopsis*.
[b] Three isolates could not be analyzed at the reference center because of absence of growth or contamination when received.

TABLE 2 Species isolated and number of strains by study period (October versus May)

Species	No. (%) of strains		
	October 2010	May 2011	Total
Aspergillus fumigatus	98 (47.6)	58 (50.0)	156 (48.5)
Aspergillus flavus	18 (8.74)	9 (7.76)	27 (8.39)
Aspergillus terreus	18 (8.74)	8 (6.90)	26 (8.07)
Aspergillus tubingensis	21 (10.2)	1 (0.86)	22 (6.83)
Aspergillus niger	17 (8.25)	4 (3.45)	21 (6.52)
Aspergillus nidulans	5 (2.43)	3 (2.59)	8 (2.48)
Rhizopus arrhizus	6 (2.91)	1 (0.86)	7 (2.17)
Scedosporium boydii	1 (0.49)	5 (4.31)	6 (1.86)
Aspergillus species[a]	9 (4.37)	9 (7.76)	17 (5.28)
Scedosporium species[b]	4 (1.94)	5 (4.31)	9 (2.80)
Penicillium species[c]	1 (0.49)	5 (4.31)	6 (1.86)
Fusarium species[d]	1 (0.49)	3 (2.59)	4 (1.24)
Mucorales species[e]	4 (1.94)	1 (0.86)	5 (1.55)
Other[f]	3 (1.46)	4 (3.45)	7 (2.17)
Total	206 (100.00)	116 (100.00)	322 (100.00)

[a] The *Aspergillus* species isolated included *A. alliaceus*, *A. calidoustus*, *A. carneus*, *A. fumigatiaffinis*, *A. insuetus*, *A. keveii*, *A. lentulus*, *A. sygowii*, *A. viridinutans*, *A. weterdijkiae*, and *N. pseudofischeri*.
[b] The *Scedosporium* species isolated included *S. apiospermum*, *S. aurantiacum*, and *S. prolificans*.
[c] The *Penicillium* species isolated included *P. chrysogenum*, *P. glabrum*, *P. cetrinum*, and *P. minioluteum*.
[d] The *Fusarium* species isolated included *F. oxysporum*, *F. proliferatum*, and *F. solani*.
[e] The Mucorales species isolated included *Lichtheimia ramosa*, *L. corymbifera*, *Rhizopus microsporus*, and *Rhizomucor pusillus*.
[f] The other species isolated included *Arthrinium* species, *Cladosporium* species, *Eupenicillium javanicum*, *Phialemonium curvatum*, *Psathyrella candolleana*, *Purpureocillium lilacinum*, and *Scopulariopsis brevicaulis*.

evance in 8/12 cases (66%) and as colonizers in the other 4. This difference was close to statistical significance ($P = 0.04$). Of the 13 cases of proven infections, most were caused by *Aspergillus* species but Mucorales isolates were found in two cases of sino-orbital infections, *Fusarium oxysporum* was isolated from the blood of a cirrhotic patient suffering from fungemia, and *Scopulariopsis brevicaulis* was isolated from a cardiac valve.

The species distribution is showed in Table 2. The most frequent species was *Aspergillus fumigatus* with 156 isolates (48.5%), followed by *Aspergillus flavus* with 27 isolates (8.4%), *Aspergillus terreus* with 26 isolates (8.1%), *Aspergillus tubingensis* with 22 isolates (6.8%), and *Aspergillus niger* with 21 isolates (6.5%). The rest of the species had fewer than 10 isolates. The low number of isolates of most of the species precludes statistical analysis, but some findings can be noted. First, some species were more frequently considered to be of clinical relevance than to be colonizers, but only in the cases of *A. terreus*, *A. nidulans*, and *Rhizopus arrhizus* (synonym, *Rhizopus oryzae*) was that difference significant ($P < 0.01$). Table 2 also shows the distribution of fungal species by study period (October versus May). Some species, such as *A. tubingensis*, *A. niger*, and *R. arrhizus*, were more frequently isolated in October 2010 than in May 2011, unlike *Aspergillus calidoustus*, *Aspergillus alliaceus*, *Scedosporium boydii*, and *Scedosporium apiospermum*, which were collected more commonly in May 2011. In order to avoid bias from centers, an analysis of species distribution by participant was done. No significant differences were observed, and outbreaks due to a specific species in the study periods were not reported. *A. fumigatus* was the most common fungal species in all of the participants, followed by other *Aspergillus* species, *Scedosporium* species, and members of the order Mucorales.

The identification of organisms by PCR amplification and DNA sequencing allowed us to detect cryptic or sibling fungal species (Table 2). Regarding complexes of *Aspergillus* species, of the total of 278 *Aspergillus* isolates, 40 (14.5%) were classified as cryptic species. The *Aspergillus* section *Fumigati* included 162 strains of which 6 (3.7%) were non-*A. fumigatus sensu stricto*, i.e., 3 of *Aspergillus lentulus*, 1 of *Aspergillus viridinutans*, 1 of *Aspergillus fumigatiaffinis*, and 1 of *Neosartorya pseudofischeri*. *Aspergillus* section *Flavi* was represented by 30 strains, 27 of *A. flavus* and 3 of *A. alliaceus*. *Aspergillus* section *Nigri* included 22 *A. tubingensis* and 21 *A. niger* strains. *Aspergillus* section *Terrei* included 26 *A. terreus* strains and 1 *Aspergillus carneus* strain. *Aspergillus* section *Nidulantes* included 8 *A. nidulans* strains. Other *Aspergillus* sections, such us *Usti* (4 *A. calidoustus* strains, 1 *Aspergillus insuetus* strain, and 1 *Aspergillus keveii* strain), *Versicolores* (1 *Aspergillus sydowii* strain), and *Circumdati* (1 *Aspergillus westerdijkiae* strain), were also represented.

Table 3 shows the geometric mean MICs, MIC ranges, MIC_{50}s (MICs causing inhibition of 50% of the isolates tested), MIC_{90}s, and MIC modes for the species isolated in this study. Only data for species represented by three or more isolates are displayed. *In vitro* resistance was uncommon among the most frequently recovered species. According to EUCAST breakpoints, resistance to amphotericin B was found in 35/322 (10.8%) isolates, resistance to itraconazole was found in 32/322 (10%), resistance to voriconazole was found in 36/322 (11.2%), and resistance to posaconazole was found in 41/322 (12.7%). *In vitro* resistance was more common among rare and emerging species, and multiresistant isolates were isolated in some cases.

No *A. fumigatus* isolate was resistant *in vitro* to amphotericin B, itraconazole, and voriconazole. One *A. fumigatus* strain showed a MIC of posaconazole of 0.50 mg/liter. Other taxa belonging to the genus *Aspergillus* showed some level of resistance *in vitro* (Table 4). Four (14.8%) of 27 *A. flavus* and 7 (27%) of 26 *A. terreus* isolates were resistant to amphotericin B. Of the 40 cryptic/sibling strains of *Aspergillus* species complexes, 16 (40%) were resistant *in vitro* to at least one antifungal compound. All of the *A. lentulus* strains were resistant to itraconazole, all of the *A. calidoustus*

TABLE 3 Antifungal susceptibility testing results

Species (no. of isolates), parameter	MIC (mg/ml)[a] of:								
	AMB	ITC	VRC	PSC	RVC	TRB	CPF	MCF	ANF
Aspergillus fumigatus (156)									
GM[b]	0.26	0.17	0.49	0.50	0.05	2.90	0.36	0.03	0.03
MIC_{50}	0.25	0.25	0.50	0.50	0.06	4.0	0.25	0.03	0.03
MIC_{90}	0.50	0.25	1.0	1.0	0.12	8.0	1.0	0.06	0.03
Mode	0.25	0.25	0.5	0.5	0.06	4.0	0.25	0.03	0.03
Range	0.06–1.0	0.12–1.0	0.12–2.0	0.25–1.0	0.015–0.50	0.06–16.0	0.06–2.0	0.03–0.50	0.03–0.06
Aspergillus flavus (27)									
GM	1.50	0.24	0.66	0.90	0.09	0.12	2.70	1.50	1.30
MIC_{50}	1.0	0.25	0.50	1.0	0.12	0.12	1.0	32.0	32.0
MIC_{90}	8.0	1.0	1.0	2.0	0.12	0.50	32.0	32.0	32.0
Mode	1.0	0.25	0.50	1.0	0.12	0.06	32.0	32.0	32.0
Range	0.50–32.0	0.06–1.0	0.12–4.0	0.25–4.0	0.015–0.25	0.03–4.0	0.25–32	0.03–32	0.03–32
Aspergillus terreus (26)									
GM	1.62	0.12	0.92	0.62	0.05	0.17	1.0	0.04	0.05
MIC_{50}	1.0	0.12	1.0	0.50	0.06	0.12	1.0	0.03	0.03
MIC_{90}	4.0	0.25	2.0	1.0	0.12	0.25	4.0	0.06	0.06
Mode	1.0	0.12	1.0	0.50	0.06	0.12	2.0	0.03	0.03
Range	0.50–8.0	0.06–0.25	0.5–2.0	0.25–2.0	0.015–0.12	0.12–0.50	0.12–32.0	0.03–32.0	0.03–32.0
Aspergillus tubingensis (22)									
GM	0.11	0.42	0.76	1.13	0.09	0.26	0.32	0.05	0.03
MIC_{50}	0.12	0.50	1.0	2.0	0.12	0.25	0.50	0.03	0.03
MIC_{90}	0.12	1.0	2.0	2.0	0.12	0.50	1.0	0.12	0.03
Mode	0.12	0.50	1.0	2.0	0.12	0.50	0.50	0.03	0.03
Range	0.06–0.12	0.03–32.0	0.25–2.0	0.25–2.0	0.03–0.25	0.03–2.0	0.06–2.0	0.03–0.25	0.03–0.06
Aspergillus niger (21)									
GM	0.18	0.36	0.70	0.91	0.09	0.13	0.36	0.04	0.03
MIC_{50}	0.12	0.50	1.0	1.0	0.12	0.12	0.50	0.03	0.03
MIC_{90}	0.50	0.50	1.0	2.0	0.12	0.50	1.0	0.12	0.03
Mode	0.12	0.50	1.0	1.0	0.12	0.06	0.5	0.03	0.03
Range	0.06–1.0	0.06–1.0	0.25–2.0	0.25–2.0	0.015–0.25	0.03–0.50	0.06–1.0	0.03–0.25	0.03–0.03
Aspergillus nidulans (8), range	0.12–32.0	0.06–0.50	0.12–1.0	0.12–0.25	0.03–0.12	0.12–0.50	0.5–32.0	0.03–0.50	0.03–0.06
Rhizopus arrhizus (7), range	0.12–1.0	0.25–16.0	4.0–16.0	0.25–8.0	0.25–1.0	32.0–32.0	0.25–32.0	0.03–32.0	0.03–32.0
Scedosporium boydii (6), range	4.0–32.0	0.50–16.0	0.25–16.0	1.0–16.0	0.50–16.0	32.0–32.0	0.5–16.0	0.12–0.25	0.25–1.0
Aspergillus calidoustus (4), range	0.50–1.0	1.0–8.0	4.0–4.0	4.0–4.0	2.0–4.0	0.50–0.50	0.03–0.50	0.03–0.06	0.03–0.03
Scedosporium apiospermum (4), range	2.0–16.0	16.0–16.0	1.0–16.0	8.0–16.0	2.0–16.0	32.0–32.0	2.0–32.0	0.03–32.0	0.25–32.0
Scedosporium prolificans (4), range	32.0–32.0	16.0–16.0	16.0–16.0	16.0–16.0	16.0–16.0	32.0–32.0	8.0–32.0	32.0–32.0	32.0–32.0
Aspergillus alliaceus (3), range	16.0–32.0	0.03–0.12	0.25–0.50	0.25–0.50	0.015–0.12	0.03–0.50	0.12–32.0	0.03–0.06	0.03–0.12
Aspergillus lentulus (3), range	1.0–8.0	4.0–4.0	2.0–2.0	1.0–2.0	0.06–0.12	0.25–0.50	0.25–32.0	0.03–32.0	0.03–32.0

[a] AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; RVC, ravuconazole; PSC, posaconazole; TRB, terbinafine; CPF, caspofungin; MCF, micafungin. ANF, anidulafungin.
[b] GM, geometric mean.

strains were resistant to voriconazole and posaconazole, and all of the *A. alliaceus* strains were resistant to amphotericin B.

Regarding other fungal species, *Scedosporium* species showed high MICs of most of drugs tested. *S. prolificans* was clearly a multiresistant species, but some *S. boydii* and *S. apiospermum* strains showed susceptibility *in vitro* to voriconazole and posaconazole and in some cases to echinocandins (Table 3). *Fusarium* species were resistant *in vitro* to all of the azole agents and echinocandins. Amphotericin B showed some *in vitro* activity against some *Fusarium* isolates. Five different species belonging to the order Mucorales were found (*R. arrhizus*, *Lichtheimia ramosa*, *Lichtheimia corymbifera*, *Rhizopus microsporus*, and *Rhizomucor pusillus*) and only amphotericin B showed good activity against all of these species. Echinocandins had no activity against these fungal species. Of the azoles, posaconazole showed moderate activity against all of the species (MIC_{50}s, ≤0.5 mg/liter), voriconazole showed high MICs, and itraconazole was active against *Lichtheimia* isolates (MIC, 0.5 mg/liter). Seven strains of *Penicillium* were isolated, and amphotericin B and echinocandins showed activity against all of them, while the azoles showed variable results, with *Penicillium cetrinum* and *Penicillium minioluteum* isolates being resistant to azoles *in vitro*.

TABLE 4 *Aspergillus* species strains resistant to amphotericin B, itraconazole, voriconazole, and posaconazole *in vitro*

Species (no. of isolates)	No. (%)[a] with:			
	AMB MIC > 2 mg/liter	ITC MIC > 2 mg/liter	VRC MIC > 2 mg/liter	PSC MIC > 0.25 mg/liter
A. fumigatus (156)	0	0	0	1 (0.6)
A. flavus (27)	4 (14.8)	0	0	0
A. terreus (26)	7 (27)	0	0	0
A. tubingensis (22)	0	1 (4.5)	0	0
A. niger (21)	0	0	0	0
A. nidulans (8)	1 (12.5)	0	0	0
A. calidoustus (4)	0	2 (50)	4 (100)	4 (100)
A. alliaceus (3)	3 (100)	0	0	0
A. lentulus (3)	1 (33.7)	3 (100)	0	0
A. sydowii (1)	0	0	0	1 (100)
A. carneus (1)	0	0	0	0
N. pseudofischeri (1)	0	0	0	0
A. viridinutans (1)	0	0	0	0
A. fumigatiaffinis (1)	1 (100)	1 (100)	0	0
A. insuetus (1)	1 (100)	1 (100)	1 (100)	1 (100)
A. westerdijkiae (1)	1 (100)	0	0	0
A. keveii (1)	0	1 (100)	1 (100)	1 (100)
Total (277)	19 (6.8)	10 (3.6)	6 (2.2)	8 (2.9)

[a] AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; PSC, posaconazole.

Finally, terbinafine was active *in vitro* against *Aspergillus* species apart from *A. fumigatus*. Other fungal species were not susceptible to this compound.

DISCUSSION

The FILPOP study is the first Spanish population-based survey of the prevalence of fungal species and antifungal drug resistance. The design of this study included the molecular identification of organisms by DNA target sequencing to detect cryptic/sibling species and susceptibility testing by the EUCAST reference method (29).

The use of molecular methods in fungal studies has produced several changes in fungal taxonomy. New species have been described, and others have been discovered to be complexes of several species. In addition, the emergence of resistance in fungal infections seems to be increasing (21, 22). These changes in taxonomy and the emergence of resistant strains have produced a need for strain identification and susceptibility testing, and several studies with the aim of species reclassification have been conducted (32–34).

First among the difficulties in planning a survey of invasive mold diseases is the limitation of collecting samples from cases of proven infections. Mold species are saprophytes of humans and laboratory contaminants, and thus, their isolation in cultures is not of clinical interest in many cases. The results of the FILPOP study show that the prevalence of isolation of filamentous fungi in cultures of clinical samples from deep sites is low (0.016 to 0.017/1,000 inhabitants). That prevalence is very similar, regardless of the time of year (spring or fall), although some variations by participant were found, as evidenced by the fact that 6 (20%) of the 29 centers that took part in the survey did not isolate any organisms during the study periods. Researchers reported more than 60% of the isolates tested as colonizers or without clinical relevance, and only 13 cases were proven infections.

The species distribution in the FILPOP study proves that *Aspergillus* species are still the most common molds isolated from human samples from deep sites (>85%). Emerging pathogens are not as rare as previously suggested, since they were isolated from 14% of the samples tested. *Scedosporium* species were found in 5% of the cases, Mucorales species were found in 3.7%, and *Penicillium* and *Fusarium* species were found in 2 and 1.2%, respectively.

A. fumigatus represented less than 50% of the isolates recovered, and the other 16 species of this genus were isolated. Regarding *A. flavus*, *A. terreus*, and *A. niger*, regional differences in the presence of these species in clinical samples have been reported; thus, *A. flavus* has been described as the most common species of *Aspergillus* isolated in some centers (35) and *A. terreus* is particularly frequent in Austria (36). Balajee et al. (37), analyzing *Aspergillus* strains from a multicenter study of transplant patients performed in the United States, found a higher rate of *A. flavus* isolation (13.3 over 9.7%), comparable to that of *A. niger* (6.0 versus 7.6%) and lower than that of *A. terreus* (5.0 versus 9.4%).

The number of rare and cryptic/sibling species belonging to the *Aspergillus* complexes found in the FILPOP study is interesting. Rare *Aspergillus* species (others than *A. fumigatus*, *A. flavus*, *A. terreus*, and *A. niger*) accounted for >30% of the isolates found, and cryptic species (those identifiable by DNA sequencing only) were isolated in 12% of the cases. The correct characterization of those cryptic species could have clinical relevance, as many of them (40% of the strains analyzed) showed *in vitro* resistance to all of the currently used antifungal agents. A similar result was found in the study performed by Balajee et al. (37). Among the cryptic species found in the FILPOP study, the most frequent ones were *A. tubingensis* (section *Nigri*) with 22 isolates (7.9%), *A. calidoustus* (section *Usti*), with 4 isolates (1.4%), and *A. lentulus* (section *Fumigati*), with 3 isolates (1.1%). Interestingly, the number of isolates of *A. tubingensis* in this study was higher than that of its sibling species *A. niger*.

Regarding other species of filamentous fungi, members of the genera *Scedosporium* and *Fusarium* and the order Mucorales have been described as emerging pathogens (1, 2, 7, 38). In this study, the second most frequent genus was *Scedosporium*, accounting for almost 5% of the isolates. Classically, *S. apiospermum* and *S. prolificans* have been described as the only species of this genus able to cause human infections; however, several taxonomic studies (5, 39) have proven that these species are complexes of taxa. In the FILPOP study, six isolates of *S. boydii*, four each of *S. apiospermum* and *S. prolificans*, and one of *S. aurantiacum* were found. The presence of *S. prolificans* is particularly relevant because of its multiresistant nature (18), and while it is almost absent from most countries, it has been found to be more prevalent in some regions, such as Australia and Spain (18, 40).

The species of the order Mucorales and the genus *Fusarium* play increasingly important roles in immunocompromised patients (7, 41, 42). Their prevalence in this study was low, compared with that in other studies, which have reported frequencies of Mucorales infection of 7 to 10% and *Fusarium* infection of 2 to 5% (17, 43). Two *Rhizopus* species (*R. arrhizus* and *R. microsporus*) were found in this study. *R. arrhizus* was the most frequent species of the order Mucorales (six strains), but *Lichtheimia* and *Rhizomucor* were also found, while no *Mucor* isolates were identified.

The emergence of resistance has been described in some European countries, and exposure to azoles in patients (21) or in the environment (22) is a possible explanation for this emergence. However, the FILPOP study shows that resistance is uncommon among the most frequently isolated species. Nevertheless, resistance to azoles was found in cryptic species of *Aspergillus*. All of the species of *Aspergillus* section *Usti* were found to be resistant to azole drugs, as previously reported (44). Resistance to azoles was also found among *Aspergillus* section *Fumigati* isolates, a fact that has also been investigated (3, 33) and has been associated with therapeutic failures in some cases of aspergillosis (21, 45). High MICs were also observed for *A. tubingensis* of *Aspergillus* section *Nigri*, in accordance with the results reported by Alcazar-Fuoli et al. (34).

Resistance to amphotericin B was found in some isolates of *A. terreus*, as described before (46). This resistance is especially important, because infections caused by *A. terreus* have been associated with a lower response rate and a poorer outcome (36). Resistance to polyenes was also found in 14% of the *A. flavus* isolates found. Resistance to these drugs in *A. flavus* has being described in several papers (47, 48). In agreement with previous studies (49), all of the isolates of *A. alliaceus* were also classified as resistant.

The results of the FILPOP study show that cryptic/sibling *Aspergillus* species are more prevalent in clinical samples than are other species of filamentous fungi regarded as emerging species (belonging to the order Mucorales and the genera *Scedosporium* and *Fusarium*). The frequency of cryptic species is high, standing at 12% of the cases analyzed. The identification of those species is clinically relevant, since antifungal drug resistance is common (40%) in isolates of those sibling/cryptic taxa. Consequently, the correct identification and susceptibility testing of fungal species are increasingly important. For a routine laboratory, we advise that strains implicated in deep infections be tested for antifungal susceptibility to assess their drug susceptibility profiles and then sent to the reference center for correct classification and epidemiological analysis. Multicenter studies should be performed to evaluate the incidence of these cryptic species in other geographic areas and elaborate therapeutic guidelines according to the results.

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